TITLE: METHOD OF INHIBITING DIHYDROFOLATE REDUCTASE; SCREENING ASSAY FOR THE IDENTIFICATION OF NOVEL THERAPEUTICS AND THEIR CELLULAR TARGETS

FIELD OF THE INVENTION

The present invention relates to the use of compounds identified as inhibitors of *Escherichia coli* dihydrofolate reductase (DHFR), the mechanism of action of said compounds being confirmed using an assay of the present invention. The invention also relates to a screening assay for the identification of novel antibacterial, antifungal, antiparasitic and anticancer therapeutics and their cellular targets and to methods of treating diseases using agents identified using the assay.

BACKGROUND OF THE INVENTION

One of the most significant hurdles in target-based drug discovery is that of the gap between *in vitro* potency in the inhibition of the function of a protein and efficacy against the target cell. With modern biochemical and medicinal chemical tools of lead generation and lead optimization, drug discoverers can rightly expect that given the appropriate time and resources, very potent compounds can be found for a purified protein target. This is because the physical principles for the development of potency against a protein target are, to a great extent, understood. Potency against the purified target, however, is only one of the requirements of efficacy in target-based drug discovery. A more daunting hurdle, particularly for antimicrobial drug discovery, is the development compounds that can penetrate living cells to reach intracellular targets. Unlike those principles for the development of potent lead compounds against protein targets, our understanding of natural laws governing access and interaction of a given compound to the intracellular space of a bacterial, fungal or even human cell are poorly understood.

For many years researchers have exploited the effect of gene dosage and protein expression in molecular genetic studies of resistance to chemotherapeutic agents. Such studies have been particularly beneficial to

the identification of resistance genes for compounds with antibacterial (1-4), antifungal (5-8), anti-parasitic (9, 10) and anticancer (11, 12) properties. From these studies it is understood that overexpression of a protein target will often lead to resistance to the chemotherapeutic agent owing to two general mechanisms. In one mechanism, perhaps the most common, overexpression of a protein involved in the modification or efflux of the chemotherapeutic agent leads to resistance. Alternatively overexpression of the protein target itself often also leads to resistance. The latter facilitates the identification of genes that are the targets of agents of unknown mechanism.

In view of the foregoing, there is a need in the art to develop screening assays that allow the simultaneous identification of novel antibacterial, antifungal, antiparasitic or anticancer agents and their cellular targets.

SUMMARY OF THE INVENTION

The present inventors have used a high-throughput *in vitro* screening assay to identify agents with inhibitory activity against *Escherichia coli* dihydrofolate reductase (DHFR). Accordingly, the present invention relates to a method of inhibiting DHFR comprising administering to an animal in need thereof, an effective amount of a compound selected from one or more of:

a compound of Formula I;

a compound of Formula II;

any one of compounds 1-11 as shown in Table 1; and

pharmaceutically acceptable salts and hydrates of a compound of Formula I, a compound of formula II and compounds 1-11.

Further, the invention includes the use of a compound selected from one or more of:

a compound of Formula I;

a compound of Formula II;

any one of compounds 1-11 as shown in Table 1; and

pharmaceutically acceptable salts and hydrates of a compound of Formula I, to inhibit DHFR in an animal in need thereof, as well as the use of a compound selected from one or more of:

a compound of Formula I;

a compound of Formula II;

any one of compounds 1-11 as shown in Table 1; and

pharmaceutically acceptable salts and hydrates of a compound of Formula I, to prepare a medicament to inhibit DHFR in an animal in need thereof.

In an embodiment of the invention the DHFR is bacterial DHFR, in particular *E. coli* DHFR.

The present inventors have also developed a robust system to simultaneously identify potential therapeutic agents and the cellular targets of the agents. The method exploits principles of target overexpression and drug resistance for the development of a high throughput screening method for the identification of the therapeutic agents and their targets. Using this system, it was confirmed that the antibacterial activity of the compounds presented above is related to their capacity to inhibit DHFR.

Accordingly, the present invention provides a method for identifying a candidate therapeutic agent and a cellular target molecule that is modulated by the agent comprising:

(a) contacting a plurality of test agents with a first target cell;

(b) selecting test agents from step (a) that inhibit the growth of the first target cell, wherein said selected test agents are candidate therapeutic agents;

(c) contacting a candidate therapeutic agent identified in step (b) with (i) the first target cell and separately with (ii) a second target cell that overexpresses one or more genes;

(d) comparing the growth of the first target cell with the second target cell wherein the inhibition of growth of the first target cell and not the second target cell indicates that the second target cell overexpresses the cellular target molecule of the candidate therapeutic; and, optionally

(e) isolating the cellular target molecule.

The present invention provides a method for identifying a candidate therapeutic agent and a cellular target molecule that is modulated by the agent comprising:

(a) contacting a candidate therapeutic agent with (i) a first target cell and separately with (ii) a second target cell that overexpresses one or more genes;

(b) comparing the growth of the first target cell with the second target cell wherein the inhibition of growth of the first target cell and not the second target cell indicates that the second target cell overexpresses the cellular target molecule of the candidate therapeutic; and, optionally

(c) isolating the cellular target molecule.

The present invention also extends to any candidate therapeutic agents and cellular target molecules identified using the above assays.

The present invention also includes a kit for use in identifying candidate therapeutic agents and their cellular targets comprising the first and the second target cells and instructions for the use thereof.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows plasmid maps of two different clones selected from the genomic library for *ddlA*.

Figure 2 is a graph showing a duplicate screen of 1000 Maybridge compounds for growth inhibition of *E. coli* MC1061. Statistical analysis of the screening data established a Z-factor (13) of 0.42. A threshold for active molecules of three standard deviations from the mean corresponded to optical density values of 0.37 (indicated by the box in the bottom left corner of the graph). Fifteen actives were identified from this duplicate screen.

Figure 3 shows a replicate plot of the screen of 50,000 small molecules against *E. coli* DHFR. Compounds that perturbed *E. coli* DHFR activity to three standard deviations below the high control mean in both replicates (Hit Zone) were deemed to be active against DHFR, and were selected for secondary screening.

Figure 4 are graphs showing the IC_{50} analysis for competitive inhibition. Data are shown for DHFR with a known DHF-competitive inhibitor, Trimethoprim, (panel A) and apparent competitive inhibitor **9** (panel B). Plots for IC_{50} determination are shown at 30 μM DHF (O) and 100 μM DHF (●). IC_{50} values were extracted from assay data using nonlinear regression analysis (SigmaPlot 8.0 software, SPSS Science, Chicago, IL) of the equation $v = a\,(1 - [I]/(IC_{50} + [I])) + c$, where v is the reaction rate, a is the amplitude of inhibition, [I] is the inhibitor concentration and c is residual activity at infinite inhibitor concentration.

Figure 5 is a schematic showing the model of DHFR binding by **6** and **7** (inset) into the *E. coli* DHFR active site, in the presence of NADPH (PDB code 1RX3) (33). Modeling is based upon the structure of **1** bound to *C. albicans* DHFR (PDB code 1IA1) (31), and was constructed using SYBYL 6.8 with the Biopolymer module (Tripos Inc., St Louis, MO.).

Figure 6 is a graph showing the dependence of the MIC on arabinose concentration for EB492 in Luria Bertani media. Open circles are tetracycline and closed circles are trimethoprim.

Figure 7 are graphs showing the dependence of the MIC on arabinose concentration for EB492 in Luria Bertani media. Open circles are tetracycline and closed circles are the molecule indicated.

DETAILED DESCRIPTION OF THE INVENTION

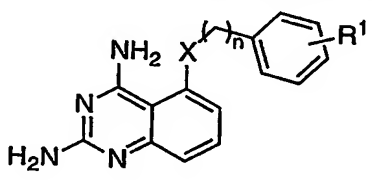
I. Dihydrofolate Reductase Inhibitory Compounds

Dihydrofolate reductase (DHFR) is a well-characterized enzyme (EC 1.5.1.3) that catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). Tetrahydrofolate is an important cofactor for a number of one carbon transfer reactions and is essential for the biosynthesis of purines, pyrimidines, several amino acids (15). One of the

most significant consequences of inhibition of this enzyme is thymidylate deficiency leading to the disruption of DNA synthesis. Thus DHFR has long been recognized as a drug target for a wide range of diseases including cancer (16), malaria (17) and bacterial infections (18). Trimethoprim has found particular clinical utility as an inhibitor of DHFR that shows striking selectivity for the bacterial enzymes over that from the human host (19). Clinical resistance to trimethoprim has, however, limited its use to all but a few therapeutic indications (20). A high-throughput screen of *Escherichia coli* DHFR using a diverse, high-quality library of compounds was performed in order to identify novel inhibitors of the bacterial enzyme.

Using a high throughput screening assay described in greater detail hereinbelow, 11 compounds were identified as competitive inhibitors of DHFR. The structures of the 11 compounds are shown in Table 1. Four of these 11 molecules were evaluated for their antibacterial efficacy against a laboratory strain of *E. coli* and against the same strain that was overexpressing recombinant *E. coli* DHFR. The minimum inhibitory concentration (MIC) for all of these molecules showed a dependence on the expression of DHFR in this latter strain, which is consistent with the conclusion that the antibacterial activity of these molecules is related to their capacity to inhibit bacterial DHFR. This is further support for the ability of the assay of the present invention to identify therapeutic agents and cellular target molecules that are modulated by the agents.

In light of the identification of the 11 compounds shown in Table 1 as inhibitors of bacterial DHFR, the present invention further relates to a method of treating conditions that benefit from an inhibition of DHFR, particularly bacterial DHFR, comprising administering to an animal in need thereof, an effective amount of a compound selected from one or more of a compound of Formula I, and pharmaceutically acceptable salts and solvates thereof:



I

wherein

R^1 is selected from the group consisting of C_{1-4}alkyl, halo and CF_3;

X is O or S; and

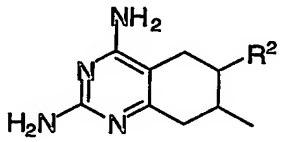
n is 0 or 1.

The present invention also relates to the use of a compound selected from a compound of Formula I as defined above, and pharmaceutically acceptable salts and hydrates thereof, to treat conditions that benefit from an inhibition of DHFR, particularly bacterial DHFR, as well as the use of a a compound selected from a compound of Formula I as defined above, and pharmaceutically acceptable salts and hydrates thereof, to prepare a medicament to treat conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR.

The group "R^1" may be located at any position on the phenyl ring. In embodiments of the invention, R^1 is located at the position para to the "X" substituent.

In specific embodiments of the invention, the compound of Formula I is selected from one or more of compounds 1, 2, 3, 4 and 5 as shown in Table 1, and pharmaceutically acceptable salts, solvates or hydrates thereof.

Further the present invention relates to a method of treating conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR, comprising administering to an animal in need thereof, an effective amount of a compound selected from one or more of a compound of Formula II and pharmaceutically acceptable salts and solvates thereof:



II

wherein R^2 is selected from the group consisting of H and C_{1-4}alkyl.

The present invention also relates to the use of a compound selected from one or more of a compound of Formula II as defined above, and pharmaceutically acceptable salts and solves thereof, to treat conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR, as well as the

use of an effective amount of a compound selected from one or more of a compound of Formula II as defined above, and pharmaceutically acceptable salts and solvates thereof, to prepare a medicament to treat conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR.

In specific embodiments of the invention, the compound of Formula II is selected from one or more of compounds 6 and 7 as shown in Table 1, and pharmaceutically acceptable salts and solvates thereof.

Finally the present invention further relates to a method of treating conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR, comprising administering to an animal in need thereof, an effective amount a compound selected from one or more of compounds 8 to 11 as shown in Table 1, and pharmaceutically acceptable salts and solvates thereof. In embodiments of the invention the compound is compound 9 as shown in Table 1, and pharmaceutically acceptable salts and solvates thereof.

The present invention also relates to the use of a compound selected from one or more of compounds 8 to 11 as shown in Table 1, and pharmaceutically acceptable salts and solvates thereof, to treat conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR, as well as the use of a compound selected from one or more of compounds 8 to 11 as shown in Table 1, and pharmaceutically acceptable salts and solvates thereof, to prepare a medicament to treat conditions that benefit from an inhibition of DHFR, in particular bacterial DHFR. In embodiments of the invention the compound is compound 9 as shown in Table 1, and pharmaceutically acceptable salts and solvates thereof.

By inhibiting DHFR, the compounds may be used to treat any condition in which inhibition of this enzyme provides a desirable effect, for example, cancer, malaria and bacterial infections.

In particular, the condition that benefits from an inhibition of DHFR is bacterial infection. Accordingly, the present invention also relates to a method of treating bacterial infections comprising administering an effective amount of a compound selected from one or more of:

(a) a compound of Formula I, as defined hereinabove;

(b) a compound of Formula II, as defined herein above;

(c) a compound selected from compounds 8-11 as shown in Table 1; and

(d) pharmaceutically acceptable salts and solvates of (a), (b) and (c),

to a cell or animal in need thereof. In specific embodiments of the invention, the compound of Formula I is selected from one or more of compounds 1-5 as shown in Table 1 and the compound of Formula II is selected from one or more of compounds 6 and 7 as shown in Table 1.

Further, the present invention includes the use of a compound selected from one or more of:

(a) a compound of Formula I, as defined hereinabove;

(b) a compound of Formula II, as defined herein above;

(c) a compound selected from compounds 8-11 as shown in Table 1; and

(d) pharmaceutically acceptable salts and solvates of (a), (b) and (c),

to treat bacterial infections, or to prepare a medicament or pharmaceutical composition to treat bacterial infections.

The bacteria may be any bacteria whose growth is affected by the inhibition of DHFR. In an embodiment of the invention the bacteria are, for example, *E. coli, Bacillus Subtilis, Streptococci, Staphylococci, Enterococci, Salmonella, Haemophilus influenza, Pseudomonas aeruginosa, Bacillus anthracis* and *Helicobacter pylori.* In a further embodiment of the invention the bacterial infection is an *E. coli* infection.

The compounds of Formulae I and II, and compounds 1-11, may also be used as tools, for example, in *in vitro* screening assays for inhibitors of DHFR, or in any such assay where inhibition of DHFR is desired. In such assays, the compound may be labeled, for example, with a radioactive label or fluorescent label. Accordingly, the present invention also relates to a method of inhibiting DHFR *in vitro* comprising administering an effective amount of a compound selected from one or more of:

(a) a compound of Formula I, as defined hereinabove;

(b) a compound of Formula II, as defined herein above;

(c) a compound selected from compounds 8-11 as shown in Table 1; and

(d) salts and solvates of (a), (b) and (c),

to a cell or assay mixture.

The present invention further relates to the use of a compound selected from one or more of:

(a) a compound of Formula I, as defined hereinabove;

(b) a compound of Formula II, as defined herein above;

(c) a compound selected from compounds 8-11 as shown in Table 1; and

(d) salts and solvates of (a), (b) and (c),

to inhibit DHFR *in vitro*.

In embodiments of the invention, the *in vitro* assay involves bacterial DHFR. In further embodiments the bacteria are for example, *E. coli, Bacillus Subtilis, Streptococci, Staphylococci, Enterococci, Salmonella, Haemophilus influenza, Pseudomonas aeruginosa, Bacillus anthracis* and *Helicobacter pylori*. In a further embodiment of the invention the bacteria are *E. coli*.

The compounds of Formulae I and II, and compounds 1-11, are either commercially available or may be prepared using standard procedures known to a person skilled in the art. Compounds 1-11 were purchased from Maybridge (Cornwall, England). The structure of compound 9 (Table 1) was incorrectly identified by the company. Exhaustive nuclear magnetic resonance (NMR) experiments have confirmed the structure to be as shown in Table 1. The compound and its biological activity remain the same.

The formation of solvates of these compounds will vary depending on the compound and the solvate. In general, solvates are formed by dissolving the compound in the appropriate solvent and isolating the solvate by cooling or using an antisolvent. The solvate is typically dried or azeotroped under ambient conditions.

The term an "effective amount" or a "sufficient amount " of an agent as used herein is that amount sufficient to effect beneficial or desired results, including clinical results, and, as such, an "effective amount" depends upon

the context in which it is being applied. For example, in the context of administering an agent for inhibiting DHFR, an effective amount of an agent is, for example, an amount sufficient to achieve a reduction in DHFR activity as compared to the response obtained without administration of the agent.

As used herein, and as well understood in the art, "treating" or "treatment" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" or "treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

"Palliating" a disease or disorder means that the extent and/or undesirable clinical manifestations of a disorder or a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not treating the disorder. In the context of treating a bacterial infection, palliating may, for example, refer to the inhibition or reduction of the infection.

The "inhibition" or "suppression" or "reduction" of a function or activity, such bacterial infection, is to reduce the function or activity when compared to otherwise same conditions except for a condition or parameter of interest, or alternatively, as compared to another conditions.

The term "animal" as used herein includes all members of the animal kingdom including human. The animal is preferably a human.

The term "a cell" as used herein includes a plurality of cells. Administering a compound to a cell includes *in vivo, ex vivo* and *in vitro* treatment.

The term "C_{1-4}alkyl" as used herein means straight and/or branched chain alkyl groups containing from one to four carbon atoms and includes methyl, ethyl, propyl, isopropyl, t-butyl and the like.

The term "halo" as used herein means halogen and includes chloro, flouro, bromo, iodo and the like.

The term "pharmaceutically acceptable" means compatible with the treatment of animals, in particular, humans.

The term "pharmaceutically acceptable salt" means an acid addition salt which is suitable for or compatible with the treatment of patients.

The term "pharmaceutically acceptable acid addition salt" as used herein means any non-toxic organic or inorganic salt of any base compound of the invention, or any of its intermediates. Basic compounds of the invention that may form an acid addition salt include those having a basic nitrogen, for example NH_2. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulfuric and phosphoric acids, as well as metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids that form suitable salts include mono-, di-, and tricarboxylic acids such as glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, benzoic, phenylacetic, cinnamic and salicylic acids, as well as sulfonic acids such as p-toluene sulfonic and methanesulfonic acids. Either the mono or di-acid salts can be formed, and such salts may exist in either a hydrated, solvated or substantially anhydrous form. In general, the acid addition salts of the compounds of the invention are more soluble in water and various hydrophilic organic solvents, and generally demonstrate higher melting points in comparison to their free base forms. The selection of the appropriate salt will be known to one skilled in the art. Other non-pharmaceutically acceptable salts, e.g. oxalates, may be used, for example, in the isolation of the compounds of the invention, for laboratory use, or for subsequent conversion to a pharmaceutically acceptable acid addition salt.

The term "solvate" as used herein means a compound wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. Examples of suitable solvents are ethanol, water and the like. When water is the solvent, the molecule is referred to as a "hydrate".

The compounds may be examined for their efficacy in inhibiting DHFR, in particular bacterial DHFR, using any known assay, or, for example, the assay described in Example 2 hereinbelow. The compounds may also be examined for their efficacy in inhibiting bacterial infection using any known assay, for example by monitoring the growth of the bacteria in the presence of the compounds and comparing to controls.

The compounds of Formulae I and II, and compounds 1-11, or salts, hydrates or solvates thereof, are preferably formulated into pharmaceutical compositions for administration to human subjects in a biologically compatible form suitable for administration *in vivo*.

The compositions comprising an effective amount of compounds of Formula I, Formula II or compounds 1-11, or salts, hydrates or solvates thereof, can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

In accordance with the methods of the invention, compounds of Formula I, Formula II or compounds 1-11, or salts, hydrates or solvates thereof, may be administered to a subject in a variety of forms depending on the selected route of administration, as will be understood by those skilled in the art. The compounds or compositions may be administered, for example, by oral, parenteral, buccal, sublingual, nasal, rectal, patch, pump or transdermal administration and the pharmaceutical compositions formulated accordingly. Parenteral administration includes intravenous, intraperitoneal, subcutaneous, intramuscular, transepithelial, nasal, intrapulmonary,

intrathecal, rectal and topical modes of administration. Parenteral administration may be by continuous infusion over a selected period of time.

Compounds of Formula I, Formula II or compounds 1-11, or salts, hydrates or solvates thereof, may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsules, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the compound may be incorporated with excipient and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like.

Compounds of Formula I, Formula II or compounds 1-11, or salts, hydrates or solvates thereof, may also be administered parenterally or intraperitoneally. Solutions of the compound can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, DMSO and mixtures thereof with or without alcohol, and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. A person skilled in the art would know how to prepare suitable formulations. Conventional procedures and ingredients for the selection and preparation of suitable formulations are described, for example, in Remington's Pharmaceutical Sciences (1990 - 18th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersion and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists.

Compositions for nasal administration may conveniently be formulated as aerosols, drops, gels and powders. Aerosol formulations typically comprise a solution or fine suspension of the active substance in a physiologically acceptable aqueous or non-aqueous solvent and are usually presented in single or multidose quantities in sterile form in a sealed container, which can

take the form of a cartridge or refill for use with an atomising device. Alternatively, the sealed container may be a unitary dispensing device such as a single dose nasal inhaler or an aerosol dispenser fitted with a metering valve which is intended for disposal after use. Where the dosage form comprises an aerosol dispenser, it will contain a propellant which can be a compressed gas such as compressed air or an organic propellant such as fluorochlorohydrocarbon. The aerosol dosage forms can also take the form of a pump-atomizer.

Compositions suitable for buccal or sublingual administration include tablets, lozenges, and pastilles, wherein the active ingredient is formulated with a carrier such as sugar, acacia, tragacanth, or gelatin and glycerine. Compositions for rectal administration are conveniently in the form of suppositories containing a conventional suppository base such as cocoa butter.

The dosage of compounds of Formula I, Formula II or compounds 1-11, or salts, hydrates or solvates thereof, can vary depending on many factors such as the pharmacodynamic properties of the compound, the mode of administration, the age, health and weight of the recipient, the nature and extent of the symptoms, the frequency of the treatment and the type of concurrent treatment, if any, and the clearance rate of the compound in the animal to be treated. One of skill in the art can determine the appropriate dosage based on the above factors. The compounds may be administered initially in a suitable dosage that may be adjusted as required, depending on the clinical response.

Compounds of Formula I, Formula II or compounds 1-11, or salts, hydrates or solvates thereof, can be used alone or in combination with other agents that treat bacterial infections or in combination with other types of DHFR inhibitors.

II. Screening Assay

The present inventors have developed a robust system to simultaneously identify agents with antibacterial activity and the cellular targets of these agents. This method exploits principles of target

overexpression and drug resistance for the development of a high throughput screening method for the identification of therapeutic compounds and their targets. Using this method, the present inventors have confirmed that one of the cellular targets for the compounds described herein above is the *folA*, the gene encoding DHFR.

Proof of principle for the assay of the invention has been achieved with the isolation of genes encoding the targets of three antibiotics whose mechanisms are well understood (i.e., *murA* for fosfomycin, *ddlA* for cycloserine, and *folA* trimethoprim). A preliminary screen of 1000 small molecules revealed 5 drug-like molecules that were growth inhibitory to *E. coli* and lead to the isolation of a resistance gene for one of those 5 molecules that was a well characterized was a multidrug efflux transporter (*acrB*). Based on the proof of principle and preliminary screens, on-going expression screens will likewise identify both targets and resistance genes for novel antibacterial molecules. Downstream secondary screens will unequivocally identify novel target-antibacterial compound pairs. This screening assay is generalizable to a wide variety of systems including the discovery of novel antifungal, antiparasitc and anticancer molecules and their targets.

Accordingly, the present invention provides a method for identifying a candidate therapeutic agent and a cellular target molecule that is modulated by the agent comprising:

(a) contacting a plurality of test agents with a first target cell;

(b) selecting test agents from step (a) that inhibit the growth of the first target cell, wherein said selected test agents are candidate therapeutic agents;

(c) contacting a candidate therapeutic agent identified in step (b) with (i) the first target cell and separately with (ii) a second target cell that overexpresses one or more genes;

(d) comparing the growth of the first target cell with the second target cell wherein the inhibition of growth of the first target cell and not the second target cell indicates that the second target cell overexpresses the cellular target molecule of the candidate therapeutic; and, optionally

(e) isolating the cellular target molecule.

The present invention also provides a method for identifying a candidate therapeutic agent and a cellular target molecule that is modulated by the agent comprising:

(a) contacting a candidate therapeutic agent with (i) a first target cell and separately with (ii) a second target cell that overexpresses one or more genes;

(b) comparing the growth of the first target cell with the second target cell wherein the inhibition of growth of the first target cell and not the second target cell indicates that the second target cell overexpresses the cellular target molecule of the candidate therapeutic; and, optionally

(c) isolating the cellular target molecule.

The term "a cell" as used herein includes more than one cell or a plurality of cells.

The first target cell can be any cell to which one wishes to generate a therapeutic agent including, but not limited to, bacteria, fungus, parasites and cancer cells. In one embodiment, the target cell is a bacterial target including model organisms such as *Escherichia coli* and *Bacillus subtilis*; and pathogens such as *Streptococci*, *Staphylococci*, *Enterococci*, *Salmonella*, *Haemophilus influenza*, *Pseudomonas aeruginosa*, *Bacillus anthracis* and *Helicobacter pylori*.

In one embodiment of the invention, each test agent in step (a) is administered at a different concentration in order to determine the minimal inhibitory concentration (MIC) of each test compound. Once the inhibitory concentration of a compound is known, such a concentration can be used in step (c) when contacting the agent with the first and second target cells.

The second target cell will be the same type of cell as the first target cell but will be transformed to overexpress one or more genes present in the first target cell. In one embodiment, the second target cell is transformed with a multicopy random genomic library that will allow the overexpression of all of the genes present in the first target cell.

In an alternative strategy to avoid selecting for multidrug efflux pumps the second cell line containing the genomic selection pool might be engineered to avoid cloning efflux pumps. One example of how the second cell line could be engineered to this end would be the construction of an *E. coli* strain deficient in the gene *tolC*, a gene known to express a protein having a role in enabling either AcrAB or AcrEF to function as efflux pumps in bacteria (14).

In yet another strategy to avoid selecting for multidrug efflux pumps the second cell line containing the genomic selection pool might be derived from sub-pools that have been shown not to contain clones that overexpress troublesome efflux pumps. The genomic selection pool, for example, may contain some 20,000 clones in total but is derived from 20 subpools of 1,000 clones. Each of the subpools could be screened using a test compound known to select for efflux pumps such as *acrAB* and *acrEF*. Subpools that are devoid of clones overexpressing efflux pumps could then be mixed to generate diverse and nearly comprehensive genomic libraries but that do not contain clones overexpressing efflux pumps.

The test agents that may be used can be any agent which one wishes to test including, but not limited to, proteins, peptides, nucleic acids (including RNA, DNA, antisense oligonucleotide, peptide nucleic acids), carbohydrates, organic compounds, natural products, library extracts, and other samples that one wishes to test for therapeutic activity against a particular target. In one embodiment, the test agents are from a small molecule library.

The method is adaptable to high-throughput screening applications. For example, a high-throughput screening assay may be used which comprises any of the methods according to the invention wherein aliquots of the target cells are exposed to a plurality of test compounds within different wells of a multi-well plate. The method of the invention may be "miniaturized" in an assay system through any acceptable method of miniaturization, including but not limited to multi-well plates, such as 24, 48, 96 or 384-wells per plate, micro-chips or slides. The assay may be reduced in size to be conducted on a micro-chip support, advantageously involving smaller

amounts of reagent and other materials. Any miniaturization of the process which is conducive to high-throughput screening is within the scope of the invention.

II. Uses of the Assay

The present invention includes all possible uses of the screening assay of the invention, some of which are summarized below.

(a) Therapeutic Agents and Targets

The invention extends to any agents or targets identified using the screening method of the invention. Once a potential therapeutic agent is identified using the screening method of the invention, one of skill in the art can readily conduct further tests to prove the therapeutic potential of the agent. One can also further study the targets to further elucidate their role in the disease process.

The invention also includes a pharmaceutical composition comprising a therapeutic agent identified using the screening method of the invention in admixture with a suitable diluent or carrier. The invention further includes a method of preparing a pharmaceutical composition for use in therapy comprising mixing a therapeutic agent identified according to the screening assay of the invention with a suitable diluent or carrier.

(b) Kits

The development of the screening assay of the invention allows the preparation of kits for use in identifying novel therapeutic agents and their targets. The kits would comprise the reagents suitable for carrying out the methods of the invention, packaged into suitable containers and providing the necessary instructions for use. For example, the kit may comprise both the first and the second target cells for use in the assay of the invention. In the specific screen of the invention, the kit may contain a plurality of target cells, each overexpressing a particular gene product(s). The kit may provide instructions for preparing the appropriate target cells as well as instructions for carrying out the assay of the invention.

The term "instructions" or "instructions for use" typically includes a description describing the reagent concentration or at least one assay method

parameter such as the relative amount of the reagent-sample admixtures, temperature, conditions and the like.

Accordingly, the present invention provides a kit for use in identifying a therapeutic agent and its cellular target comprising a first target cell to which one wishes to generate a therapeutic agent and a second target cell that overexpresses one or more genes present in the first target cell.

(c) Therapeutic Uses

The assay and kit of the invention allow the identification of novel therapeutic agents that may be used in developing drugs for treating or preventing many diseases and conditions. Such diseases and conditions include, but are not limited to, bacterial, parasitic and fungal infections as well as cancer. Accordingly, the present invention also provides a method of treating a disease comprising administering an effective amount of a therapeutic agent isolated according to the method of the invention to an animal in need thereof.

The term "effective amount" as used herein is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. The effective amount of a compound of the invention may vary according to factors such as the disease state, age, sex, and weight of the animal. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The term "animal" as used herein includes all members of the animal kingdom, including humans. Preferably, the animal to be treated is a human.

(d) Drug Discovery

The present invention also includes all business applications of the screening assay of the invention including conducting a drug discovery business.

Accordingly, the present invention also provides a method of conducting a drug discovery business comprising:

(a) providing one or more assay systems for identifying a potential therapeutic agent;

(b) conducting therapeutic profiling of agents identified in step (a), or further analogs thereof, for efficacy and toxicity in animals; and

(c) formulating a pharmaceutical preparation including one or more agents identified in step (b) as having an acceptable therapeutic profile.

In certain embodiments, the subject method can also include a step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

The present invention also provides a method of conducting a target discovery business comprising:

(a) providing one or more assay systems for identifying a potential therapeutic agent;

(b) (optionally) conducting therapeutic profiling of agents identified in step (a) for efficacy and toxicity in animals; and

(c) licensing, to a third party, the rights for further drug development and/or sales for agents identified in step (a), or analogs thereof.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

In the example described here, the inventors have exploited the principles of target overexpression and drug resistance in the development of a high throughput screening method for the identification of antibacterial compounds and their targets. The inventors are currently applying this method to the discovery of novel antibacterial agents and their targets in the model bacterium *E. coli*. The method is generalizable to a wide variety of systems including antifungal, antiparasitc and anticancer drug discovery.

General Method

The method begins with the identification of compounds in a small molecule screening library that have antibacterial activity against *E. coli* strain

MC1061 a hyper-permeable rough lipopolysaccharide mutant (21). The inventors have chosen a hyper-permeable strain of *E. coli* in order to maximize the opportunity to detect small molecules with antibacterial potential. Each of the compounds that demonstrate antibacterial activity are subsequently subjected to MIC analysis to determine the minimum concentration necessary to inhibit bacterial growth. Having established growth-retarding concentrations of each active molecule the inventors then set about to search for the cellular target of the antibacterial compound using a multicopy genomic library of *E. coli* that has been transformed into strain MC1061. Selections for growth from this pool of clones on growth-inhibitory concentrations of an active compound will select for clones that overexpress the target protein. Indeed, the inventors outline proof of principle in this Example with the identification in the genomic library of three celebrated bacterial targets using their respective drugs. The inventors have also applied the approach to a small commercial library of screening compounds in a preliminary application of the method.

Random genomic library from *E. coli*

The random *E. coli* genomic library was constructed by Deborah Siegele at Texas A&M University. The library was made by cloning approximately 3 to 4 kb gel-purified fragments from a partial *Sau3AI* digest of DNA from MG1655. The fragments were cloned into the *BamHI* site of pGEM7. The library was acquired in the form of a ligation mix that was subsequently transformed into *E. coli* strain MC1061 (*hsdR mcrB araD*139 D(*araABC-leu*)7679 Δ*lacX*74 *galU galK rpsL thi*) by electroporation and plated on LB agar selecting for streptomycin (ST, 50 µg/mL) and ampicillin (AP, 50 µg/mL). Some 20, 000 colonies were then tooth-picked from these plates after overnight growth (37°C) such that each clone was transferred to a single well in a 96-well plate containing 200 µL LB-ST-AP broth and grown overnight with shaking at 37°C. Each overnight culture (125 µL) was transferred to a well in a deep-well polypropyiene "stock plate" containing 125 µL of 30% glycerol in LB broth. The stock plate was sealed and stored at -80°C. Each overnight culture (50 µL) was also mixed with an equal volume of 30% glycerol in LB

broth and transferred to a "screening pool" such that the pool contained all 20,000 clones. The "screening pool" was stored in aliquots at -80°C and is, as the name suggests, the pool from which the inventors attempt to identify resistance clones. The "stock plate" is a source of the each of the clones for future use.

Proof of principle with known antibiotics.

As proof of principle the inventors first attempted to select for the genes encoding the targets of three well-known antibiotics using the expression screening method. In a typical experiment the inventors systematically exposed untransformed MC1061 and MC1061 transformed with the genomic library (the screening pool) to increasing concentrations of the following three antibiotics: fosfomycin, trimethoprim and D-cycloserine. More precisely, the inventors grew *E. coli* strain MC1061 overnight in broth LB-ST, diluted 10^3-fold in LB-ST and plated about 10^4 bacteria (100 µL per plate) on LB agar-ST with increasing concentrations (0.1, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 µg/mL) of each of the antibiotics. In parallel the inventors plated a similar number of bacteria (10^4) from the screening pool on such plates. In each case the inventors isolated colonies from the screening pool that were resistant to concentrations of these antibiotics that were lethal to MC1061. Table 1 summarizes the results for each of these where 7, 20 and 8 clones were isolated that were resistant to growth inhibitory concentrations of fosfomycin (320 µg/mL), trimethoprim (2.5 µg/mL) and D-cycloserine (160 µg/mL). The inventors used PCR to test for the presence of the expected target gene in the plasmids carried by a subset of these and determined that 3 of 7 clones resistant to fosfomycin harbored a plasmid that contained the gene *murA*. For trimethoprim, 5 of 5 clones contained *folA* in high copy and for D-cycloserine 6 of 6 contained *ddlA*. Sequence analysis of the inserts into pGEM7 confirmed for all those tested that the expected target gene was present in clones selected for using each of these antibiotics. Figure 1 shows the maps of two different clones identified that contained the gene *ddlA*.

A first screen against library of 1000 compounds.

In addition to proof of principle work using well-characterized antibiotics the inventors have also done a preliminary screen against a 1000 compound, non-proprietary commercial library from Maybridge (Cornwall, England). First the inventors set out to identify compounds with activity against *E. coli* by screening for inhibition of growth. *E. coli* strain MC1061 was grown overnight in broth LB with streptomycin (ST, 50 µg/mL), diluted 10^7-fold in LB-ST and deposited into 96-well microwell plates (200 µL/well). To each test well 10 µL of screening compound (1 mM in DMSO) was added and the plate incubated for 36 hours at 37°C with shaking (150 rpm) before reading the optical density (600 nm). Figure 2 shows the results of duplicate screens of MC1061 against these 1000 compounds. The result of this duplicate screen was the identification of 15 compounds with statistically significant antibacterial activity.

Of the 15 compounds, the inventors focused on five that appeared to be most drug-like (22) and might be reasonable leads for a medicinal chemistry program (Table 2). The inventors re-acquired these compounds from the supplier (Maybridge) in quantities necessary for retesting and minimum inhibitory concentration (MIC) determination. MIC testing was on LB-ST agar. The inventors grew *E. coli* strain MC1061 overnight in broth LB-ST, diluted 103-fold in LB-ST and plated about 104 bacteria (100 µL per plate) on LB agar-ST with increasing concentrations of the compounds in Table 2. In parallel, the inventors plated a similar number of bacteria (10^4) from the screening pool on another set of plates. For compound SEW04978 the inventors isolated 3 clones from the screening pool that were resistant to a concentration of 64 µg/mL while a concentration of 32 µg/mL was lethal to MC1061. The plasmids from these three clones were subsequently purified and sequenced to determine the gene content of the inserts in the respective cloning sites. The plasmid inserts of these three clones proved to be identical and contained the complete open reading frame for a single gene *acrB*. Gene *acrB* encodes the acridine resistance pump, a protein that has been well characterized as a multidrug resistance efflux pump (23, 24). The

antibacterial action of SEW04978 is presumably abrogated by the overexpression of this cellular pump from the high copy plasmid pGEM7 resulting in resistance to an otherwise toxic molecule.

In summary the inventors believe that the isolation of a multidrug efflux transporter in this preliminary assay of just 1000 molecules further proves the principle that resistance genes can be isolated for novel antibacterial agents. The inventors expect that targets of these agents will also be among those resistance genes isolated as was demonstrated for the three antibiotics of known mechanism.

Example 2: Screening for Inhibitors of Bacterial DHFR

A library of compounds (50,000) sourced from Maybridge (Cornwall, England) were screened against recombinant *E. coli* DHFR in a highly automated format. The gene (*folA*) encoding dihydrofolate reductase (DHFR) was PCR amplified from *E.coli* MG1655 chromosomal DNA with primers, $^{5'}$-C ATC TTA CAT ATG ATC AGT CTG ATT GCG GC -$^{3'}$ and $^{5'}$- CTA CTC GAG CCG CCG CTC CAG AAT CT -$^{3'}$, containing *Nde*I and *Xho*I restriction sites (underlined), respectively. The gene was cloned lacking a stop codon into *Nde*I and *Xho*I digested pET26b to form pET26b-folA, which incorporates a C-terminal polyhistidine-tag. Polyhistidine-tagged DHFR was purified to homogeneity as described previously (25).

DHF reductase activity was assayed continuously in 96-well microplates by monitoring the decrease of NADPH at an absorbance of 340 nm (26). Assays were carried out at 25°C and performed in duplicate. The 200 µL reaction mixture contained 40 µM NADPH, 30 µM DHF, 5 nM DHFR, 50 mM Tris (pH 7.5), 0.01% (w/v) Triton and 10 mM β-mercaptoethanol. Test compounds from the screening library were added to the reaction before initiation by enzyme and at a final concentration of 10 µM. High activity controls consisted of reaction mixtures with DMSO only and low activity controls contained 1.5 µM Trimethoprim. Automation for high throughput screening included assay reagent handling in 96 well format. Compound addition, assay monitoring and plate handling were performed using a Sagian-Beckman Coulter linear track with a Biomek FX liquid handler and

SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, California, USA) integrated into Core Assay System (Beckman Coulter, Mississauga, Ontario, Canada). ActivityBase (IDBS Inc., Emeryville, CA), SARgen (IDBS Inc., Emeryville, CA) and Spotfire DecisionSite (Spotfire, Inc., Somerville, MA) were used for data analysis.

The assay data were of high quality with Z and Z' statistical scores (27) of 0.66 and 0.73, indicative of good signal to noise in the compound and control wells, respectively. Figure 3 shows a plot of the screening replicates against one another and illustrates the quality of the entire screen, where absolute replicates would lie on a perfect diagonal. All data are reported as percent residual activity relative to the average of the high controls. Active molecules were identified as those showing less than 75% residual activity, a statistical cut-off three standard deviations below the high control mean. Using this threshold 62 compounds were found to be inhibitors of DHFR, giving a primary hit rate of 0.12% over the entire screen.

In secondary screening, IC_{50} determinations were performed for actives from primary screening. This potency analysis was done at two DHF concentrations, 30 μM and 100 μM, to identify compounds that were competitive with DHF. Figure 4 illustrates sample IC_{50} curves for trimethoprim, a known inhibitor competitive with DHF (28), and the active compound **9**. The ratio of the two calculated IC_{50} values at the two concentrations of DHF was used to evaluate the competitiveness of each primary hit with respect to DHF. Using the equation $IC_{50} = K_i (1 + [S]/K_m)$ (29), where $K_{m, DHF} = 9.5$ μM (data not shown), the IC_{50} ratio of a true competitive inhibitor should equal 2.8 for determinations at 100 and 30 mM DHF. This secondary screen identified 11 inhibitors of DHFR, out of the 62 actives from primary screening, having IC_{50} ratios consistent with competitive behavior. Table 1 shows the structures of these 11 molecules along with their IC_{50} data and K_i values calculated from the two IC_{50} determinations. Compounds **1, 2, 4, 9** and **11** from Table 1 have IC_{50} values were in the nanomolar range and those for the remainder of the compounds were in the micromolar range.

Of the identified compounds the 5-arylthioquinazolines (**1** and **2**) have been previously identified as active against *Candida albicans* DHFR (30) but not bacterial DHFR. The structure of **1** bound to *C. albicans* DHFR has been published (31). Compounds **1-5** have been previously reported as modulators of serine/threonine protein kinase function (32). Based on the structure of DHFR with **1** present in the active site of *C. albicans* DHFR (31), compounds **6** and **7** were modeled into the active site of *E. coli* DHFR. Each of these compounds nicely occupies the DHF binding pocket (Figure 5). Of the remaining molecules, **9** and **11** are novel inhibitors of DHFR that have guanidine in common and are remarkably potent (K_i values of 26 and 65 nM, respectively). The structure of compound **9** was incorrectly identified by the commercial sounds. Extensive NMR experiments confirm that the structure is that shown in Table 1. Compound **10** is a quinolinone not previously reported to be active against dihydrofolate reductase.

Example 3: Antibacterial Properties of Novel DHFR Inhibitors and Mechanism of Action

Four molecules identified as inhibitors of *E. coli* DHFR in Example 2 were evaluated for their antibacterial efficacy against a laboratory strain of *E. coli* and against the same strain that was overexpressing recombinant *E. coli* DHFR. All of these molecules showed a dependence of minimum inhibitory concentration (MIC) on the expression of DHFR in this strain.

Critical to these studies was the creation of a strain of *E. coli* in which we could vary the expression of the *folA* gene and ultimately the copy number of the FolA protein. The *folA* gene was cloned into the pBAD18-Ap^r vector (34) and transformed into *E. coli* strain CW2553 contains the pAKO1 plasmid (35). Strain CW2553 is devoid of a functional chromosomal arabinose transporter while plasmid pAK01 encodes araE, an arabinose transporter under control of a tac (IPTG-inducible) promoter. This system allowed for the controlled expression of the *folA* gene.

The gene (*folA*) encoding dihydrofolate reductase (DHFR) was PCR amplified from *E.coli* MG1655 chromosomal DNA with primers, $^{5'}$-C GCT CTA GAT TTT TTT TAT CGG GAA ATC TCA ATG -$^{3'}$ and $^{5'}$- CTA AAG CTT TTA

CCG CCG CTC CAG AAT C-$^{3'}$, containing *Xba*I and *Hind*III restriction sites (underlined), respectively. The resulting PCR product was cloned into the *Xba*I and *Hind*III site of pBAD18-Ap^{r} to create pBAD18-folA that puts the expression of gene folA under the control of the arabinose promoter. Plasmid pBAD18-folA was subsequently transformed into CW2553 containing pAK01 to produce *E. coli* strain EB492. Strain EB492 was systematically exposed to test compounds and to arabinose in order to determine if there was a dependence for the MIC of each test compound on the arabinose concentration in the media. Figure 6 demonstrates the arabinose dependence of the MIC for trimethoprim, an antibacterial drug understood to target DHFR. Increasing expression of DHFR with increasing arabinose concentration leads to resistance to trimethoprim and not to tetracycline.

Using this system the mechanism of antibacterial action of 4 molecules identified in the biochemical screen of DHFR in Example 2 was investigated the (Figure 7). In each case these molecules show a dependence of the observed MIC for EB492 on the concentration of arabinose in the media. Such a dependence is consistent the conclusion that the antibacterial activity of these molecules is related to their capacity to inhibit bacterial DHFR.

Example: 4

Two molecules (compound 4 and compound 7, Table 1) identified as inhibitors of DHFR in Example 2 and as growth inhibitory to *E. coli* in Example 3, were subjected to a search for the cellular target of the antibacterial compounds using the pool of clones harbouring the random multicopy genomic library detailed in Example 1. Exposure of this pool of clones to growth-inhibitory concentrations of these compounds resulted the selection of clones containing the gene *folA*, encoding dihydrofolate reductase, for each of the compounds. This outcome is consistent with hypothesis that the cellular target of these antibacterial molecules is DHFR. Furthermore, compound 4 also produced clones that contained the gene *acrB*, encoding the multidrug efflux transporter. The ~~latter result~~ confirms that the expression screening method is capable of producing both the cellular target of an antibacterial compound and resistance genes such as *acrB*.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

1. Marquardt, J. L., Siegele, D. A., Kolter, R., and Walsh, C. T. (1992) *J Bacteriol 174*, 5748-52.

2. Chen, P., and Bishai, W. R. (1998) *Infect Immun 66*, 5099-106.

3. Chen, D. Z., Patel, D. V., Hackbarth, C. J., Wang, W., Dreyer, G., Young, D. C., Margolis, P. S., Wu, C., Ni, Z. J., Trias, J., White, R. J., and Yuan, Z. (2000) *Biochemistry 39*, 1256-62.

4. Apfel, C. M., Locher, H., Evers, S., Takacs, B., Hubschwerlen, C., Pirson, W., Page, M. G., and Keck, W. (2001) *Antimicrob Agents Chemother 45*, 1058-64.

5. Miura, N., Kaneko, S., Hosoya, S., Furuchi, T., Miura, K., Kuge, S., and Naganuma, A. (1999) *FEBS Lett 458*, 215-8.

6. Calabrese, D., Bille, J., and Sanglard, D. (2000) *Microbiology 146*, 2743-54.

7. Osherov, N., Kontoyiannis, D. P., Romans, A., and May, G. S. (2001) *J Antimicrob Chemother 48*, 75-81.

8. Masuda, C. A., Xavier, M. A., Mattos, K. A., Galina, A., and Montero-Lomeli, M. (2001) *J Biol Chem 276*, 37794-37801.

9. Delling, U., Raymond, M., and Schurr, E. (1998) *Antimicrob Agents Chemother 42*, 1034-41.

10. Cotrim, P. C., Garrity, L. K., and Beverley, S. M. (1999) *J Biol Chem 274*, 37723-30.

11. Burger, H., Capello, A., Schenk, P. W., Stoter, G., Brouwer, J., and Nooter, K. (2000) *Biochem Biophys Res Commun 269*, 767-74.

12. Parr, A. L., Myers, T. G., Holbeck, S. L., Loh, Y. J., and Allegra, C. J. (2001) *Anticancer Drugs 12*, 569-74.

13. Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) *J Biomol Screen 4*, 67-73.

14. Kobayashi *et al. J. Bacteriology* (2001) 183, 2646-2653.

15. Blakley, R. L. *The Biochemistry of Folic Acid and Related Pteridines*; North-Holland Publishing Co. Amsterdam, 1969; pp pp 219-358.

16. Huennekens, F. M. *Adv Enzyme Regul* 1994, *34*, 397-419.

17. Olliaro, P. L.; Yuthavong, Y. *Pharmacol Ther* 1999, *81*, 91-110.

18. Rollo, I. M. *CRC Crit Rev Clin Lab Sci* 1970, *1*, 565-583.

19. Baccanari, D. P.; Kuyper, L. F. *J Chemother* 1993, *5*, 393-399.

20. Huovinen, P. *Clin Infect Dis* 2001, *32*, 1608-1614.

21. Casadaban, M. J., and Cohen, S. N. (1980) *J Mol Biol 138*, 179-207.

22. Lipinski, C. A. (2000) *J Pharmacol Toxicol Methods 44*, 235-49.

23. Kawabe, T., Fujihira, E., and Yamaguchi, A. (2000) *J Biochem (Tokyo) 128*, 195-200.

24. Nikaido, H., and Zgurskaya, H. I. (2001) *J Mol Microbiol Biotechnol 3*, 215-8.

25. Tai, N.; Ding, Y.; Schmitz, J. C.; Chu, E. *Nucleic Acids Res* 2002, *30*, 4481-4488.

26. Hillcoat, B. L.; Nixon, P. F.; Blakley, R. L. *Anal Biochem* 1967, *21*, 178-189.

27. Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. *J Biomol Screen* 1999, *4*, 67-73.

28. Stone, S. R.; Morrison, J. F. *Biochim Biophys Acta* 1986, *869*, 275-285.

29. Baccanari, D. P.; Daluge, S.; King, R. W. *Biochemistry* 1982, *21*, 5068-5075.

30. Chan, J. H.; Hong, J. S.; Kuyper, L. F.; Baccanari, D. P.; Joyner, S. S. et al. *J Med Chem* 1995, *38*, 3608-3616.

31. Whitlow, M.; Howard, A. J.; Stewart, D.; Hardman, K. D.; Chan, J. H. et al. *J Med Chem* 2001, *44*, 2928-2932.

32. Tang, P. C. M., McMahon, G.; Weinberger, H.; Kutscher, B.; App, H. Patent, 1998, WO 9850370.

33. Sawaya, M. R.; Kraut, J. *Biochemistry* 1997, *36*, 586-603.

34. Guzman, L. M.; Belin, D.; Carson, M. J.; Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 1995, *177*, 4121-4130.

35. Morgan-Kiss, R. M.; Wadler, C.; Cronan, J. E., Jr. Long-term and homogeneous regulation of the Escherichia coli araBAD promoter by use of a lactose transporter of relaxed specificity. *Proc Natl Acad Sci U S A* 2002, *99*, 7373-7377.

Table 1. DHFR inhibitors found to be competitive with dihydrofolate.

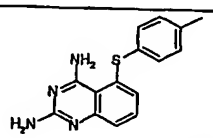
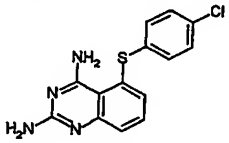
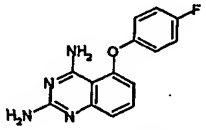
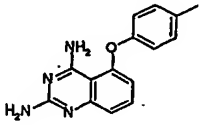
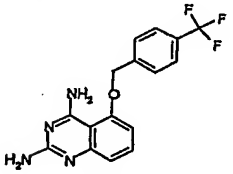
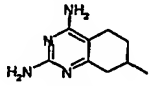
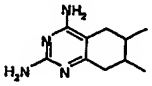
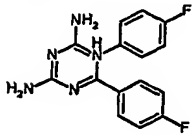
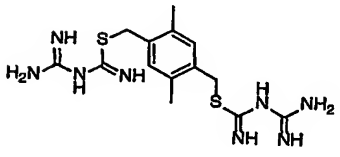
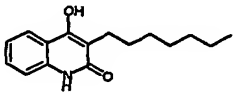
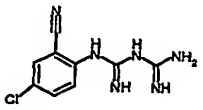
Cpd #	Structure	IC_{50} (nM) 30 µM DHF	IC_{50} (nM) 100 µM DHF	IR[1]	K_i[2] (nM)
1		310	820	2.6	73
2		320	510	1.6	61
3		400	1.0×10^3	2.6	93
4		190	420	2.3	41
5		660	1.1×10^3	1.7	130
6		1.1×10^4	2.4×10^4	2.3	2.3×10^3
7		790	2.1×10^3	2.6	190
8		1.1×10^4	1.6×10^4	1.5	2.0×10^3
9		109	302	2.8	26

Table 1 continued.

10		4.8×10^4	1.2×10^5	2.5	1.1×10^4
11		320	620	1.9	65

[1] IC_{50} ratio, (IC_{50} in the presence of 100 μM DHF) / (IC_{50} in the presence of 30 μM DHF).

[2] Inhibition constant K_i was calculated using the relationship $K_i = IC_{50} / (1 + [S]/K_m)$ (29), where [S] the substrate concentration (100 or 30 mM) and K_m was determined to be 9.5 mM (data not shown). The K_i indicated was the average of determinations at the two substrate concentrations.

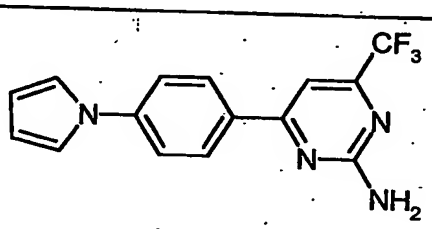
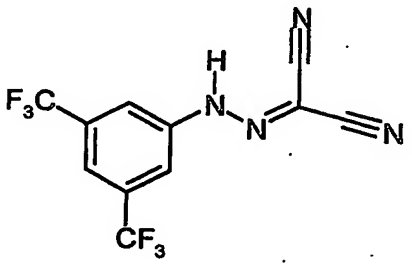
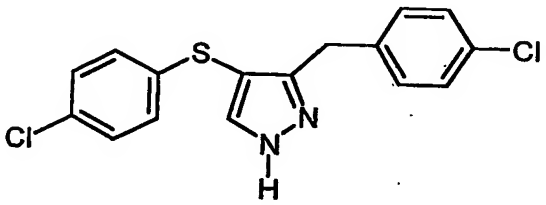
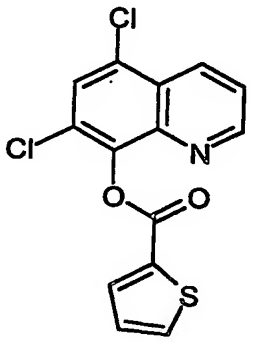
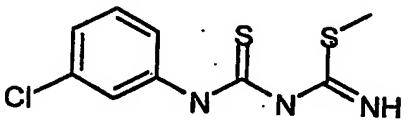
Table 2. Analysis of resistant clones isolated for known antibiotic-target pairs.

	Fosfomycin selection for *murA* 320 µg/mL	Trimethoprim selection for *folA* 2.5 µg/mL	D-cycloserine selection for *ddlA* 160 µg/mL
Colonies isolated	7	20	8
PCR verification[1]	3/7	5/5	6/6
Sequence verification[2]	3/3	2/2	2/2

[1] PCR verification involved analytical amplification by PCR of the gene of interest from a mini-prep of plasmid DNA from resistant clones. Primer pairs used in that amplification were primers that annealed to the predicted target gene and to sequences flanking the cloning site of pGEM7. Shown are the number of positive clones and the number of clones tested.

[2] Sequence verification involved sequencing of a portion of the insert of the selected clone to determine if it contained the postulated gene. Shown are the number of positive clones and the number of clones tested.

Table 3. Growth inhbitory compounds from duplicate screens of 1000 molecules.

Compound number	Structure	Growth in Primary Screens	Retest Minimum Inhibitory Concentration
BTB14887		37, 30 %	125 µg/mL
SEW04978		0.2, 0.02 %	32 µg/mL
SPB04137		2.3, 2.1 %	500 µg/mL
RH00852		2.7, 2.8 %	16 µg/mL
RJF01047		6.0, 12 %	125 µg/mL